Influence of combined genotypes of RETN G62A with C-180G and G299A polymorphisms on colon cancer risk

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Abstract: Understanding the biology and genetic alterations of adipocytokines, cell-signaling proteins secreted by adipose tissue, provides new insights into the pathogenesis and treatment of several diseases including cancer. In the present study, we investigated the role of RETN gene G62A variant in the pathophysiology of colon cancer. A group of 120 Saudi volunteers (60-colon cancer patients and 60 disease-free controls) within the same age range was studied. PCR-RELP technique was used to determine the single nucleotide polymorphism (SNP). The results were compared with the control group. Analysis of SNP+62 genotypes results showed 100% normal (GG) in both the patients and the controls. Heterozygous (GA) and homozygous (AA) genotypes were not detected in all the patients and the controls. The interesting result in this study is the combined effect of G62A SNP with RETN C-180G and G299A SNPs. The results showed a decrease risk of colon cancer reflecting the protective role of SNP G62A in the development of colon cancer. These results suggest that these variants in RETN gene and their interactions are strongly associated with the development of colon cancer.


Keywords: Resistin gene, SNPs, colon cancer, Saudi population

1. Introduction
The global burden of cancer continues to increase largely. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (WHO, 2008). Colon cancer is one of the most frequently diagnosed cancers in females and males worldwide (Parkin et al., 2005; Jemal et al., 2008; Jemal et al., 2011). Rates of colon cancer vary by race and ethnic status. In Saudi Arabia, the latest incidence report by the Saudi Cancer Registry (SCR) showed that colon cancer ranks the second most common malignancy among Saudis for all ages (10.3%) (Al-Eid et al., 2011). Researchers have indicated several risk factors that may increase a person's chance of developing colon cancer (Chan et al., 2010; Schlienger et al., 2009; Ma et al., 2013; Comstock et al., 2014). Obesity is a well-known risk factor for colon cancer, but until now, scientists were at a loss to explain why. A number of mechanisms have been proposed for the adverse effect of obesity on colon cancer risk. One of the major hypotheses is the adipocytokines, which are secreted by adipose tissue (Riondino et al., 2014; Sánchez et al., 2014). Researches have suggested that colon cancer risk rises with increasing weight and increasing the level of resistin (Kumor et al., 2009; Al-Harithy and Al-Ghafari., 2010; Gounullu et al., 2010). This finding points to a genetic reason for the link. Therefore; gaining a better understanding of the relationship between obesity and colon cancer using modern epidemiological studies that depends on genetic assays will provide new insight into the mechanisms of colon cancer pathogenesis.

Resistin, an adipocytokine, modulate metabolic and inflammatory responses (Steppan et al., 2001; Kaser et al., 2003; Bokarewa et al., 2005; Calabro et al., 2007; Filipová et al., 2009). Human resistin is expressed mainly by macrophages and induced by TNF-α (Patel et al., 2003; Lehrke et al., 2004). Resistin encoded by the RETN gene that is located on chromosome 19p13.3, and spans approximately 1,750 base pairs (Wang et al., 2002; Ghosh et al., 2003). Single nucleotide polymorphisms (SNPs) have been detected in the gene encoding human resistin as well as the promoter region (Engert et al., 2002; Bouchard et al., 2004; Mattevi et al., 2004; Norata et al., 2007; Osawa et al., 2007). Resistin gene polymorphism at position G62A reported to acts as an independent contributing factor to T2DM and hypertension (Tan et al., 2003). Krízová and his group investigated in patients with obesity, anorexia nervosa, and in control healthy normal-weight women to determine G62A contribution to metabolic phenotype and found that this SNP could contribute to metabolic phenotype of patients with obesity and anorexia nervosa (Krizová et al., 2008). In 2012, Zhangbin and co-workers
performed meta-analysis for SNP G62A and observed no significant association with obesity (Zhangbin et al., 2012). Other SNP such as G+299A has been found to have an impact on the increased resistin concentrations and might influence susceptibility to type II Diabetes Mellitus (T2DM) in Thais (Osawa et al., 2002; Suriyaprom et al., 2009). Recently, homozygous (AA) genotype at position +299 in RETN gene was found to play a role in the pathogenesis and susceptibility to obesity, impaired glucose tolerance, and T2DM in the Egyptian population as well as higher risk to develop nonalcoholic fatty acids liver disease (NAFLD) in T2DM Chinese patients (El-Shal et al., 2013; Zhange et al., 2013). In contrast, studies involving Japanese patients showed that SNP G299A in the RETN gene did not have major effects on the susceptibility to insulin resistance syndrome associated with T2DM (Ochi et al., 2003). Regarding SNP C-180G in the RETN promoter region, several studies have focused on the role of resistin in the pathophysiology of insulin resistance, obesity and metabolic syndromes. The results from those studies were controversial (Smith et al., 2003; Ochi et al., 2007; Krizová et al., 2008; Ukkola et al., 2008; Dong et al., 2012; Liu et al., 2012; Wen et al., 2013).

Given the importance of obesity in colon cancer development and the fundamental role of resistin in obesity, it is reasonable to hypothesize that RETN gene SNPs may play a role in colon cancer susceptibility. Therefore, in this study we investigated the role of RETN gene G62A variant in the pathophysiology of colon cancer. The study also evaluated the combined effect of G62A SNP with two SNPs that have previous association with resistin circulating levels, RETN C-180G and G299A.

2. Materials and methods

Study subjects

Sixty randomly selected patients with colon cancer (31 males and 29 females) and 60 age matched control subjects (30 males and 30 females) were involved in this study. All the individuals were recruited from King Abdulaziz Hospital and Oncology Center in Jeddah, Kingdom of Saudi Arabia (KSA). Healthy subjects were judged to be in good health according to their medical history and routine laboratory tests. None were taking any medication. The ethical committee of King Abdulaziz University (KAU) has approved this study. All participants provided written informed consent.

Genotyping of RETN gene polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA blood Mini kit (Qiagen, 51106). DNA samples were genotyped using PCR assays combined with restriction fragment length polymorphism (RELP). The genotype assay was used for analysis of the G299A and G62A variations. All of the PCR reactions were carried out by a Mastercycler gradient thermocycler (Eppendorf, Germany). Each 25 µl of the PCR reactions contained 2 µl genomic DNA (0.2 µg), 12.5 µl HotStar Taq Master Mix (Qiagen, 203445) in 10.1 µl RNase free water (Qiagen, 203445), and 0.2 µl of each primer (0.1 µM). The primer sequences and PCR-RFLP conditions used for SNPs G299A and G62A are mentioned in details in (Table 1 and Table 2) whereas, for SNP C-180G was published in (17, 23).

Statistical analysis

Genotype distributions, allele frequencies, odds ratio, and risk ratio between patients and controls were calculated by 2x2 contingency table. The Hardy-Weinberg equilibrium was tested for the genotypes. P-value < 0.05 was considered statistically significant.

3. Results

Genotype distribution and allele frequencies

The genotype and allele frequencies of G62A variant showed 100% normal (GG) in both patients and controls. Heterozygous (GA), and homozygous (AA) genotypes were not detected in all the patients and the controls. The genotype and allele frequencies of C-180G variant were previously examined and published (Al-Harithy et al., 2010). The genotype and allele frequencies of G299A variant were examined (Table 3). The genotypic frequencies of the patients were 5% (n=3) normal (GG), 85% (n=51) heterozygous (GA), and 10% (n=6) homozygous (AA). In controls, the results showed 25% (n=15) normal (GG), 65% (n=39) heterozygous (GA), and 10% (n=6) homozygous (AA). In patient subjects, the frequency of the G and A alleles were 47.5% and 52.5% respectively. In controls, the frequency of the G and A alleles were 57.5% and 42.5% respectively. Genotype distributions for colon cancer patients and controls were out of Hardy-Weinberg equilibrium (Goodness of fit $X^2 = 29.83$, df =1, $P =0.0001$) and (Goodness of fit $X^2= 6.55$, df=1, $P=0.01$), respectively.
Genotype combinations of the \textit{RETN} G299A and G62A polymorphisms

Genotype combinations were analyzed (GG/GG, GG/GA, GG/AA, GA/GG, GA/GA, GA/AA, AA/GG, AA/GA, and AA/AA) for G299A and G62A SNPs (Table 4). Combination between the genotypes of the two SNPs did not show a correlation with colon cancer risk except for combination between the homozygous genotype for G299A and the normal genotype for G62A (AA/GG) SNPs. The result showed non-significant decrease to the risk of colon cancer ($P=0.687$).

Genotype combinations of the \textit{RETN} C-180G and G62A polymorphisms

Genotype combinations were analyzed (CC/GG, CC/GA, CC/AA, CG/GG, CG/GA, CG/AA, GG/GG, GG/GA, and GG/AA) for C-180G and G62A SNPs (Table 5). Combination between several genotypes of the two SNPs did not show a correlation with colon cancer risk except for combination between the homozygous genotype for C-180G and the normal genotype for G62A (GG/GG) SNPs. The result showed non-significant decrease to the risk of colon cancer ($P=0.109$).

\begin{table}[h]
\centering
\caption{The primer sequences and PCR- RFLP conditions used for G299A SNP} 
\begin{tabular}{|l|l|}
\hline
\textbf{Forward primer} & (5'-GAGAGGATCCAGGAGGTCG-3')  \\
\textbf{Reverse primer} & (5'-GTGAGACCAAACGGTCCCT-3')  \\
\hline
\textbf{PCR conditions}  & 96°C for 5 min 1x \\
 & 96°C for 35 S 40x \\
 & 56°C for 35 S 40x \\
 & 72°C for 35 S 40x \\
 & 72°C for 4 min 1x \\
\hline
\textbf{Amplified product length} & 373 bp  \\
\hline
\textbf{Restriction enzyme} & \textit{AluI} (Thermo Scientific, FERFD0014) \\
\hline
\textbf{RFLP condition} & 37°C for 2 hours \\
\hline
\textbf{RFLP products length} & Normal (GG) 243 and 55 bp  \\
 & Heterozygous (GA) 243, 158, 85, and 55 bp  \\
 & Homozygous (AA) 158, 85, and 55 bp  \\
\hline
\textbf{Reference} & (9)  \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{The primer sequences and PCR- RFLP conditions used for G62A SNP} 
\begin{tabular}{|l|l|}
\hline
\textbf{Forward primer} & (5'-AGAGTCCACCGCTCTGTGTT-3')  \\
\textbf{Reverse primer} & (5'-CATCTCCAGGTTATTCCAGC-3')  \\
\hline
\textbf{PCR conditions}  & 96°C for 5 min 1x \\
 & 96°C for 35 S 40x \\
 & 55°C for 35 S 40x \\
 & 72°C for 35 S 40x \\
 & 72°C for 4 min 1x \\
\hline
\textbf{Amplified product length} & 249 bp  \\
\hline
\textbf{Restriction enzyme} & \textit{BseRI} (New England BioLabs, R0581L) \\
\hline
\textbf{RFLP condition} & 37°C for 2 hours \\
\hline
\textbf{RFLP products length} & In the presence of G allele 249 bp  \\
 & In the presence of A allele 238 and 11 bp  \\
\hline
\textbf{Reference} & (25)  \\
\hline
\end{tabular}
\end{table}
### Table 3: Genotypes and allele frequencies of RETN G299A SNP for patients and controls

<table>
<thead>
<tr>
<th>RETN polymorphism Genotypes</th>
<th>Frequencies %</th>
<th>P value</th>
<th>Odds Ratio (95% CI)</th>
<th>Risk Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients (n=60)</td>
<td>Controls (n=60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG Normal</td>
<td>5.0 (n=3)</td>
<td>25.0 (n=15)</td>
<td>1.00</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>GA Heterozygous</td>
<td>85.0 (n=51)</td>
<td>65.0 (n=39)</td>
<td>0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 (1.77 – 24.18)</td>
</tr>
<tr>
<td>AA Homozygous</td>
<td>10.0 (n=6)</td>
<td>10.0 (n=6)</td>
<td>0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0 (0.93 – 26.79)</td>
</tr>
<tr>
<td>GA + AA</td>
<td>95.0 (n=57)</td>
<td>75.0 (n=45)</td>
<td>0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.33 (1.73 – 23.23)</td>
</tr>
</tbody>
</table>

**RETN polymorphism Alleles**

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Frequencies %</th>
<th>P value</th>
<th>Odds Ratio (95% CI)</th>
<th>Risk Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>47.5</td>
<td>57.5</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>A</td>
<td>52.5</td>
<td>42.5</td>
<td>0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.49 (0.89 – 2.49)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two sided $\chi^2$ test  
<sup>b</sup> Two sided Fisher exact test

### Table 4: Genotype combinations of G299A and G62A SNPs in the RETN gene

<table>
<thead>
<tr>
<th>G299A/G62A Genotypes</th>
<th>Frequencies %</th>
<th>Odds Ratio (95% CI)</th>
<th>Risk Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/ Normal GG/GG</td>
<td>0.05 (n=3)</td>
<td>0.25 (n=15)</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>Normal/ Hetero GG/GA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal/ Homo GG/AA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hetero/ Normal GA/GG</td>
<td>0.85 (n=51)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hetero/ Hetero GA/GA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hetero/ Homo GA/AA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Homo/ Normal AA/GG</td>
<td>0.1 (n=6)</td>
<td>0.75 (n=45)</td>
<td>0.6667 (0.1481-3)</td>
<td>0.9444 (0.7507-1.1882)</td>
</tr>
<tr>
<td>Homo/ Hetero AA/GA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Homo/ Homo AA/AA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(Homo- Homozygous, Hetero- Heterozygous)  
<sup>a</sup> Two sided $\chi^2$ test  
<sup>b</sup> Two sided Fisher exact test
Table 5: Genotype combinations of SNPs C-180G and G62A in the RETN gene

<table>
<thead>
<tr>
<th>C-180G/G62A Genotypes</th>
<th>Frequencies %</th>
<th>Odds Ratio (95% CI)</th>
<th>Risk Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients (n=60)</td>
<td>Controls (n=60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal/ Normal CC/GG</td>
<td>27 (n=16)</td>
<td>0.7 (n=24)</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>Normal/ Hetero CC/GA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
<tr>
<td>Normal/ Homo CC/AA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
<tr>
<td>Hetero/ Normal CG/GG</td>
<td>55 (n=33)</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
<tr>
<td>Hetero/ Hetero CG/GA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
<tr>
<td>Hetero/ Homo CG/AA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
<tr>
<td>Homo/ Normal GG/GG</td>
<td>18.33 (n=11)</td>
<td>60 (n=36)</td>
<td>0.4583 (1.5-3.2727)</td>
<td>0.7833 (0.6-0.766)</td>
</tr>
<tr>
<td>Homo/ Hetero GG/GA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
<tr>
<td>Homo/ Homo GG/AA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 b</td>
</tr>
</tbody>
</table>

(Homo- Homozygous, Hetero- Heterozygous)

a Two sided X² test
b Two sided Fisher exact test

4. Discussion

Our study showed an interesting finding in the combined effect of the RETN G62A with G299A and C-180G SNPs on colon cancer risk. Data analysis revealed that the risk of colon cancer for patients with normal (GG) G62A genotype decreased when combined with either homozygous (AA) G299A or homozygous (GG) C-180G genotypes. Indicating that, G at single nucleotide polymorphism 62 is required for A at 299 or G at -180 in RETN gene to reduce the risk of colon cancer in Saudi patients. SNPs combination is an excellent method that gives clues for analyzing complex diseases and understanding the effect of SNPs. There have been no reports on the combination effect of the RETN G62A with G299A and C-180G SNPs on colon cancer risk to date.

There has been controversy regarding the role of resistin in humans. Polymorphisms in the gene encoding resistin are important to clarify resistin role. RETN G299A polymorphism was found to be associated with coronary artery disease (Hussain et al., 2011). Other group suggested that resistin gene polymorphisms might play an important role in pathogenesis and susceptibility to obesity, impaired glucose tolerance, and T2DM in the Egyptian population (El-Shal et al., 2013). Also in T2DM patients, Zhang and his team found an association with increases in the risk of the nonalcoholic fatty liver disease development among 299AA genotype carriers (Zhang et al., 2013). On the other hand, Tan and his group reported that G62A acts as an independent contributing factor to T2DM and hypertension (Tan et al., 2003). Krízová et al., 2008 found that SNP of the resistin gene G62A was associated with lower HbA1c in normal weight and higher cholesterol concentrations in obese group. Moreover, carriers of the minor A allele in the locus 62 of the resistin gene had significantly higher cholesterol levels than obese G/G homozygotes, while the presence of the minor A allele in healthy normal-weight women was accompanied by lower HbA1c levels. Zhangbin et al., performed meta-analysis and observed no significant association with obesity (Zhangbin et al., 2012). In addition, Boumaïza et al., failed to contribute genetic variant G62A in RETN gene with obesity and metabolic syndrome in Tunisian population (Boumaïza et al., 2012).

In the present study, we found that G299A, a SNP in the non-coding region of intron 2, increases the risk of colon cancer in colon cancer patients. Carriers of the heterozygous (GA) genotype of 299 (OR=6.5, 95%CI 1.77-24.18, P=0.002) and the homozygous (AA) genotype (OR=5.0, 95% CI 0.93-
26.79, \( P=0.11 \) had a significantly higher colon cancer risk than carriers of normal (GG) genotype. Hence, RETN 299 GA and AA variants can be used as indicators for colon cancer due to their association. Concerning C-180G, a SNP in the promoter region, we found in a previous work that this variant increases the risk of colon cancer (Al-Harithy et al., 2010). Therefore, at this point, it appears that G299A and C-180G can be considered as promising candidates for causal variants, with the possibility that certain combination with G299A and C-180G SNPs may increase or reduce colon cancer risk. To test this hypothesis, we evaluated the combined effect of RETN G62A with G299A and C-180G SNPs on the risk of colon cancer. The results showed an impact of combined effect of RETN G62A with C-180G and G299A SNPs on reducing the risk of colon cancer. These results suggest that these variants in RETN gene and their interactions are strongly associated with the development of colon cancer.

The findings in this study should be considered in light of the small number of subjects that limit the statistical power. Despite that, the design of this study is relatively strong because the controls were recruited from the same cohort as the colon cancer patients. In addition, the cases and controls have been matched by age and sex. Moreover, we evaluated the influence of polymorphisms in the RETN gene, not only based on SNP, but also on combined genotypes. The combination approach yielded a more informative relationship between the RETN and colon cancer.

In conclusion, our study provides an estimate of the RETN alleles frequencies in Saudi Arabia. It also indicates the influence of combined genotypes on colon cancer risk. The function of RETN SNPs, including interactions with other SNPs, remains to be elucidated. Further experiments will be required to clarify these points.

Competing interests:
The author declares that there are no competing interests.

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